Potential genetic causes for sexual dimorphism in immune response of *Drosophila melanogaster* in response to infection with the fungal pathogen *Beauveria bassiana*

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Abstract: The innate immune response is an organism's first line of defense against disease, be it bacterial, viral, or fungal infection, and it is an integral part of the evolutionary survival of any species. A well-studied model of innate immunity is the fruit fly *Drosophila melanogaster*, which has advanced our understanding of many immune signaling pathways that are homologous to mammals. *D. melanogaster* immune defense responds in a sexually dimorphic manner to infection with various pathogens, including infection with the fungus *Beauveria bassiana*. Specifically, females die much faster after initial infection compared to males, but the underlying genetic causes responsible are unknown. I examined potential genetic causes of the observed sexual dimorphism by testing for the presence or absence of sexual dimorphism in response to sprayed doses of *B. bassiana* in various fly strains, with mutations in Toll and IMD signaling pathways. I hypothesized that genes involved in the Toll pathway are responsible for the sexual dimorphism. The results of the experiment showed that mutations in either the Toll or IMD pathways both eliminated the sexual dimorphism. The data suggests that both pathways play important roles in the innate immune response to fungal infection in *Drosophila*, and that both pathways are required for sexual dimorphism in immune response.

Keywords: *Drosophila melanogaster* | *Beauveria bassiana* | immunity | Toll pathway | IMD pathway

Introduction:

The innate immune response is an organism's first line of defense against disease, be it bacterial, viral, or fungal infection, and it is an integral part of the evolutionary survival of any species. As such an important factor for survival, there is an enormous wealth of variability across different species for innate immune response as well as high variability within species themselves. These intraspecies differences can be caused by various factors, including environment, age, or

sex differences (Anderson et al - 1985). Sexual dimorphism in immune response is the focus of this study. A complete understanding of sexual dimorphism in human immunity, for example, might lead to the development of more individualized, potent, and accurate treatments for disease. Likewise, a similar understanding of insect immunity might lead to the development of more effective biological or chemical agents for pest control.

A well-studied model of innate immunity is the fruit fly *Drosophila melanogaster*, which has led to the understanding of immune signaling pathways that are homologous to humans' (Medzhitov et al - 1997) making this a very important model in the study of immunity. *D. melanogaster* have male female differences in immunity when infected with bacterial or fungal pathogens (Duneau et al. in preparation). When infected with the fungus *Beauveria bassiana*, females die much faster than males (Duneau et al. in preparation). However, the underlying genetic pathways that cause this sexual dimorphism remain unknown.

B. bassiana is an entomopathogenic fungus that is widely used in agriculture as an insecticide to control for several pests including termites, thrips, whiteflies, aphids and different beetles. *B. bassiana* has also been studied as a possible biological control against dengue or malaria carrying mosquitoes (Dong et al. 2011; Blanford et al. 2005). When *B. bassiana* comes into contact with an insect host, it first germinates on the cuticle before penetrating into the hemolymph, where it continues to grow, killing the host in the process, and reemerging to produce new spores.

In this study I attempt to test potential candidate genes in *Drosophila* immune pathways that might be responsible for the sexual dimorphism observed in response to infection with *B. bassiana*. I tested for the presence or absence of sexual dimorphism in survival post inoculation using various fly strains that have mutations in different genes in the Toll and IMD immune

signaling pathways. It is known in the literature that activation of both of these signaling pathways leads to the production of antimicrobial peptides, which are used to fight off infection and regulate immune response in *Drosophila* (De Gregorio et al 2002). The Toll signaling pathway has been shown to be activated by Gram (+) bacteria and fungi while the IMD pathway is known to be activated mainly by Gram (-) bacteria and some Gram (+) bacilli (Nation, 2008, Chapter 15). Due to the evidence found in the literature about fungal activation of the Toll pathway, I hypothesized that genes involved in this pathway are also responsible for male *Drosophila* having better survival outcome to fungal infection than do female; in the absence of functional genes in this pathway sexual dimorphism should not be as apparent. IMD pathway mutants on the other hand should respond to fungal infection in a similar fashion as wild-type lines and sexual dimorphism should be observed.

Methods:

Drosophila lines

A total of ten *Drosophila melanogaster* lines were inoculated with *Beauveria bassiana* in order to observe either the presence or absence of sexual dimorphism. These lines consisted of three wild-type lines, three IMD pathway mutants lines, one IMD pathway RNAi line and its paired cross control, and two Toll pathway mutant lines. The three wild-type lines used were Canton S, Oregon R, and White 1118 (w^{1118}). The three IMD pathway mutant lines were a *tak1* gene knockout, *imd* gene knockout, and relish (*rel*) gene knockout. The two Toll pathway mutant lines were a *modsp* knockout, and a Persephone (*psh*) gene knockout. The RNAi lines were obtained from a relish RNAi line crossed to a driver of fat body and hemocyte (c564 x rel RNAi) and a driver control where the driver was crossed to a white background (c564 x w^{1118}). For these crosses 5 male Vienna 49414 rel RNAi flies were crossed with 5 gal4 c564 virgin females or 5 wild-type

w^{1118} males were crossed with 5 gal4 c564 virgin females. Adults were allowed to lay eggs for two days before being dumped out of the vials. 12 days after egg the newly emerged adults were taken for the following procedure.

Handling lines prior to inoculation

Prior to inoculation, all *D. melanogaster* lines were grown and maintained in vials containing fly food (82g of glucose, 82g of brewer's yeast, and 10g of agar per liter of deionized water). Two generations before inoculation, adult flies were transferred to new vials in groups of five males and five females each, in order to collect eggs. After two days of egg collection, the adults were dumped out of the vials and the eggs were allowed to grow to adult flies. The same process was repeated for a second generation. The emerging adults were then transferred to new food vials approximately 12 days from egg in groups of five males and five females and allowed to age for another 3-4 days to have fully matured adults for the experiments. Thus, all flies were approximately 16 days from egg when used for the experiments and were assumed to be mated.

Fungal Strain and Spray Protocol

Vials containing five males and five females were separated into two groups, each with 50-60 males and 50-60 females. The control group was sprayed with 5mL of 0.03% silwet in deionized (DI) water, and treatment group was sprayed with 5mL of *B. bassiana* strains GHA suspension. The suspension was made from 0.0136g of spores in 10mL of silwet solution (0.03% silwet in DI water).

Flies were inoculated utilizing a spray tower (calibrations and specific information described in Vandenberg 1996). Prior to inoculation the flies were anesthetized with CO_2 and

placed onto an ice tray on a rotating platform inside the spray tower. Following the spray, flies were transferred to small cages with a small petri plate with fly food. Both groups were then stored in an incubator at 25°C and ~100% relative humidity on a 12:12 light:dark cycle. After 24 hours the flies were moved to a rearing room at ~25°C and 60-70% relative humidity and a 12:12 light:dark cycle where they were kept until 10 days post inoculation.

Fungus Viability Check and Spore Count

The viability of the fungus used was tested by spraying a small petri plate of fungus growing medium (2.5g yeast extract, 2.5g bactopeptone, 10g glucose, 15g agar) with approximately 1-2 spores/mm^2. This plate was then placed in an incubator at 25°C. After 24 hours the plate was examined for even distribution of growing fungus and after 3-4 days the plate was examined for a lawn of fungus.

In order to verify dosage sprayed onto the flies, a microscope cover slip was sprayed with the flies during each treatment. The cover slip was then placed into a 50 mL Falcon tube with approximately 15 small glass beads and covered with 5 mL of 0.03% silwet. The Falcon tube was shaken on a vortex shaker in order to get the spores into the solution. The suspension was then moved to another coverslip over a counting area. Using a disposable pipette, a drop of the suspension was placed onto each of the two grids of two hemocytometers. Using a light microscope, the spores in the four corner squares and center square were counted in a consistent way, as to obtain the best estimation of spores per mm^2. For this experiment, inoculations were done using a density of approximately 10^3 spores per mm^2. This dosage range was chosen since previous dosage response curves showed that at lower doses male mortality was not consistent enough to be replicable.

Survival Assay

For 10 days after treatment, mortality for males and females was recorded daily, with the exception of day 1 post infection. Deaths that occurred in the first day post handling were assumed to result from handling itself and were censored. There were similarly low levels of handling loss in the treatment and control groups. Food plates in the cages were replaced daily. Surviving adults after day 10 were also counted.

Statistical analysis

All survival data was analyzed using R version 3.1.3 (R core team 2015). Cox proportional hazards model was used to analyze the survival data and analysis of variance was used to test for the effects of replicate, sex, and replicate-by-sex interactions on survival using the model:

$$\text{Survival} \sim \text{replicate} + \text{sex} + (\text{replicate} \times \text{sex})$$

Results:

Wild Type Lines

For each of the three wild-type fly lines, Canton S, Oregon R, and w^{1118}, I recorded daily survival for ten days for males and females of control and inoculated lines (Figure 1). Analysis of the data using Cox proportional hazards model and ANOVA showed that in the untreated controls, there were no significant differences between male and female survival curves. However, in all three lines, there was a significant difference in treated male and female survival, such that females were more susceptible to fungal infection ($p < 0.002$ for Canton S, $p < 0.0001$ for Oregon R, and $p < 0.0001$ for w^{1118}). For the Canton S line there was a significant effect of replicate ($p < 0.0001$) as well as replicate-by-sex interactions ($p = 0.038$). There was also a replicate-by-sex interaction for w^{1118} ($p = 0.037$).

IMD pathway mutant lines tak1, imd, and rel

I tested three mutants of the IMD pathway, *tak1*, *imd*, and *rel*, for male and female differences in survival post inoculation (Figure 2). Analysis showed that in all three lines, there was no significant difference between male and female survival in the untreated control groups. Moreover, there was no significant difference between male and female survival in the inoculated *rel* and *imd* lines ($p = 0.061$ for *imd*, and $p = 0.43$ for *rel*). There was statistically significant difference between male and female survival in the *tak1* ($p < 0.0001$). The *imd* line did show significant difference across replicates in survival ($p = 0.045$), but there was no replicate-by-sex interaction ($p = 0.065$).

IMD pathway RNAi lines

Daily survival was measured for 10 days for control and inoculated groups of a relish RNAi line crossed to a driver of fat body and hemocyte (c564 x *rel* RNAi) and a driver control where the driver was crossed to a white background (c564 x w^{1118}) (Figure 3). Knockdown of *rel* using the RNAi line verified the results with the relish mutant line in that there was no difference between male and female survival post inoculation ($p = 0.30$). There was, however, a significant difference in survival across the replicates ($p < 0.0001$), but no replicate-by-sex interaction (0.61). For the driver control cross, there was a significant difference between male and female survival post inoculation ($p < 0.0001$).

Toll pathway mutant lines

For two Toll pathway knockout lines, *modsp* and *psh*, I recorded daily survival for ten days for males and females of control and inoculated groups (Figure 4). For the *modsp* line there was no significant difference between the sexes ($p = 0.99$), and no replicate-by-sex interaction ($p = 0.27$), but there was a significant difference in survival across replicates ($p < 0.0001$). For the *psh*

line the flies all died by day 6 after inoculation. There was no significant difference in male and female survival over 10 days ($p = 0.55$).

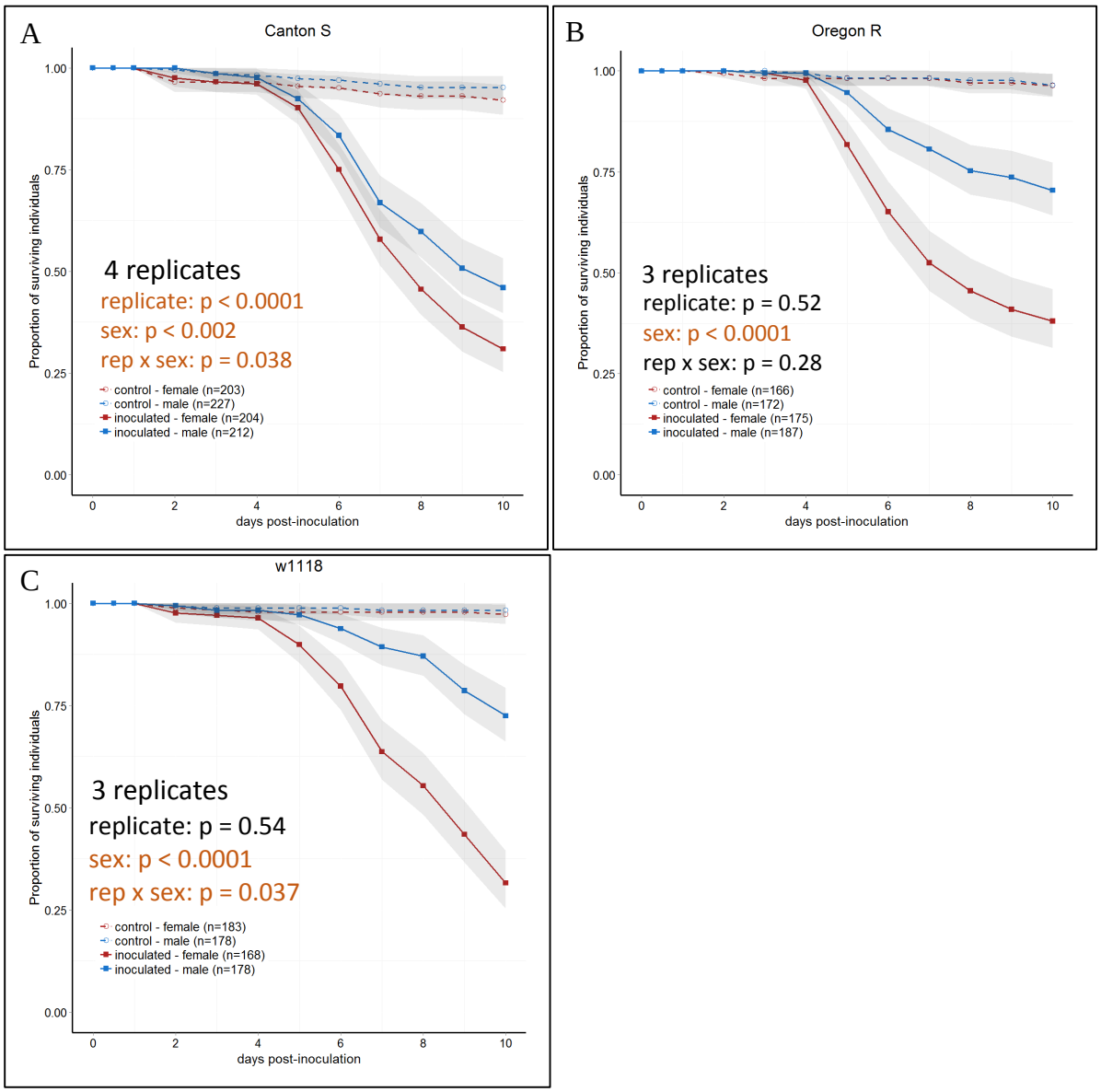

Figure 1: Ten-day survival for control and inoculated wild-type *Drosophila* lines. Control groups, represented by dashed lines, were sprayed with 5mL of 0.03% silwet in DI water and the inoculated groups, represented by solid lines, were sprayed with 5mL solution of 0.14g of *B. bassiana* suspended in 10 mL of 0.03% silwet in DI water. Male and female *Drosophila* are represented by the colors blue and red respectively. A three-way analysis of variance was used to compare survival across replicates, across sex, and replicate-by-sex interaction. The resulting p-values are shown on the graphs. n-values represent total number of flies across replicates.

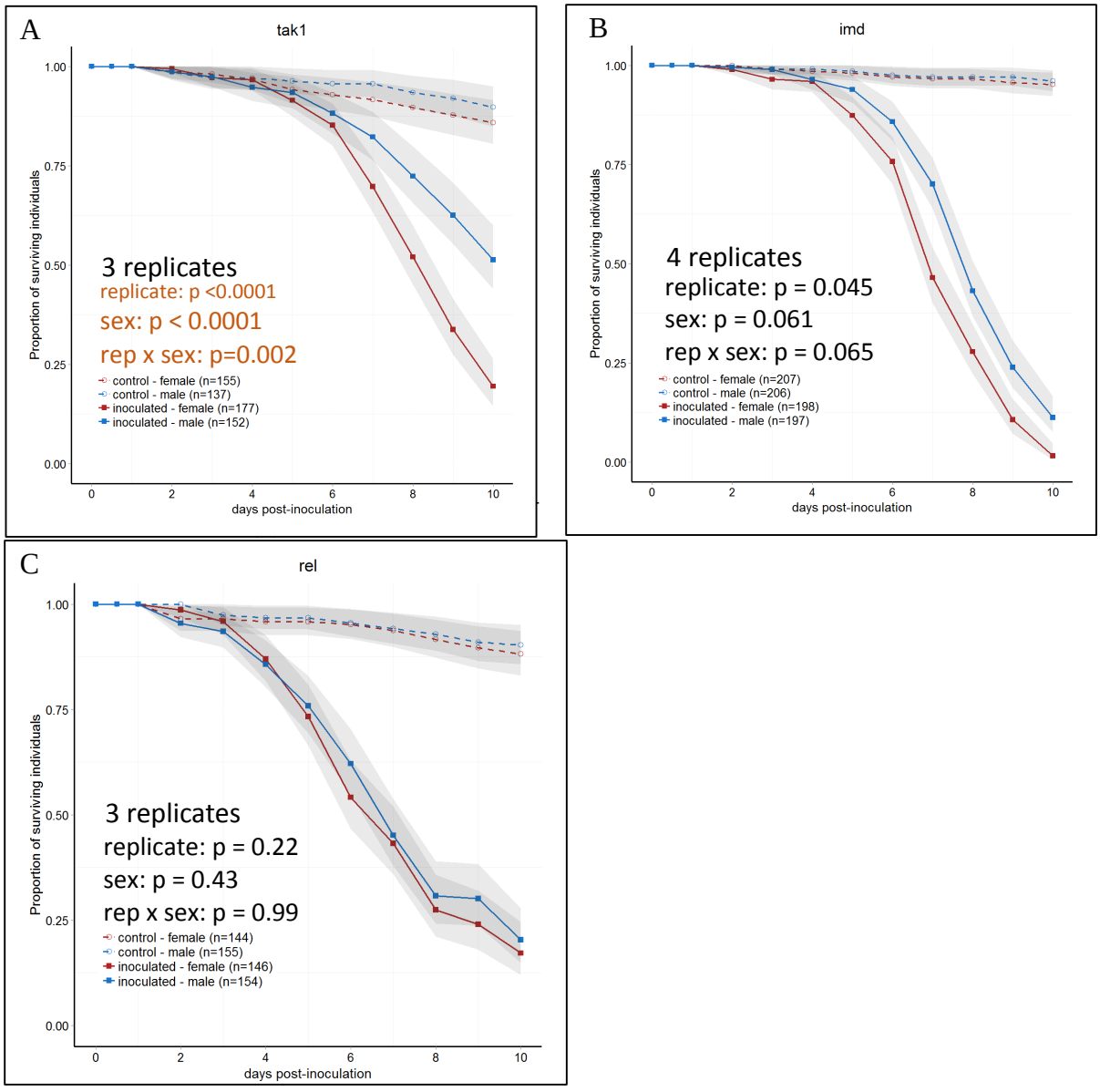

Figure 2: Ten-day survival for control and inoculated IMD pathway mutant lines. Control groups, represented by dashed lines, were sprayed with 5mL of 0.03% silwet in DI water and the inoculated groups, represented by solid lines, were sprayed with 5mL solution of 0.14g of *B. bassiana* suspended in 10 mL of 0.03% silwet in DI water. Male and female *Drosophila* are represented by the colors blue and red respectively. A three-way analysis of variance was used to compare survival across replicates, across sex, and replicate-by-sex interaction. The resulting p-values are shown on the graphs. n-values represent total number of flies across replicates.

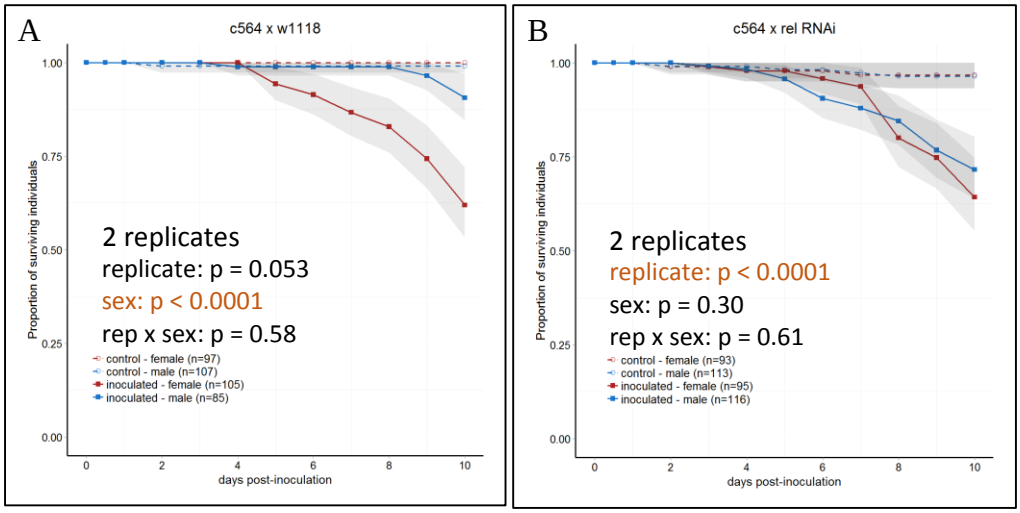

Figure 3: Ten-day survival for control and inoculated IMD pathway RNAi lines. Control groups, represented by dashed lines, were sprayed with 5mL of 0.03% silwet in DI water and the inoculated groups, represented by solid lines, were sprayed with 5mL solution of 0.14g of *B. bassiana* suspended in 10 mL of 0.03% silwet in DI water. Male and female *Drosophila* are represented by the colors blue and red respectively. A three-way analysis of variance was used to compare survival across replicates, across sex, and replicate-by-sex interaction. The resulting p-values are shown on the graphs. n-values represent total number of flies across replicates.

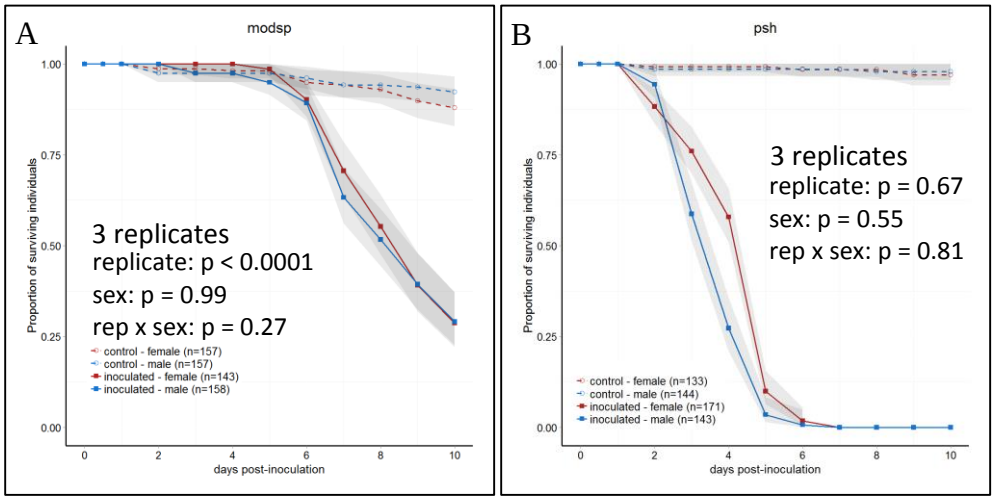

Figure 4: Ten-day survival for control and inoculated Toll pathway mutant lines. Control groups, represented by dashed lines, were sprayed with 5mL of 0.03% silwet in DI water and the inoculated groups, represented by solid lines, were sprayed with 5mL solution of 0.14g of *B. bassiana* suspended in 10 mL of 0.03% silwet in DI water. Male and female *Drosophila* are represented by the colors blue and red respectively. A three-way analysis of variance was used to compare survival across replicates, across sex, and replicate-by-sex interaction. The resulting p-values are shown on the graphs. n-values represent total number of flies across replicates.

Discussion:

All of the wild-type fly lines I tested exhibited significant difference in male and female survival over ten days after inoculation with *Beauveria bassiana,* which confirmed previous observations that females are more susceptible than males to infection with this fungal entomopathogen (Duneau et al. in preparation). I hypothesized that the Toll signaling pathway would have been responsible for *Drosophila's* sexual dimorphism in response to *B. bassiana* infection. I predicted that Toll pathway mutants would not exhibit sexual dimorphism when infected with this fungal pathogen and that IMD pathway mutants and wild-type lines would. From the results obtained in this study, my prediction was only partly correct, since both Toll pathway mutant lines showed no significant difference in male and female survival over 10 days and all wild type lines exhibited sexual dimorphism over 10 days. An unexpected result, however, was that one of the IMD mutant lines tested, relish, showed no significant differences in male and female survival over 10 days, even though the IMD pathway is not usually thought to be important in conferring resistance against fungal infection in insects.

The Toll signaling pathway is known to be activated by Gram (+) bacteria and fungi (Nation, 2008, Chapter 15), and my results agree with the notion that the Toll pathway is important to combat fungal infection in *Drosophila*. Mutations in either the *modsp* or *psh* in the Toll pathway, eliminated the sexual dimorphism in survival post inoculation. One interesting result however, was that all the *psh* mutants died within 6 days of inoculation with *B. bassiana*, where all other lines did not begin to show mortality until approximately 4-5 days post inoculation. Before observing that inoculated *psh* mutants begin to die just 2-3 days post inoculation, we had assumed that the *B. bassiana* spores that were used in inoculations required a few days before penetrating the insect cuticle. We had thought that the 4-5 day delay in inoculated mortality

reflected the time that it took the fungus to germinate and penetrate the *Drosophila* cuticle. The survival crash in the *psh* line suggests that cuticle penetration might be occurring earlier than what we had thought. A previous study has reported that mutations in the *psh* gene block induction of the Toll pathway by fungi, which resulted in greater susceptibility to fungal infection (Ligoxygakis, 2002). Currently in our lab we are in the process of replicating the experiment with the same mutant and wild-type lines studied, but are introducing *B. bassiana* to the host via micro injections as opposed to spray, bypassing the cuticle in the process, in order to see how survival results are affected. One other experiment that might provide information about *B. bassiana's* mechanism of action would be the use of a GFP-tagged *B. bassiana* line and fluorescence microscopy to monitor the progression of fungal infection in the *Drosohpila* host. This experiment would also help to confirm or deny our previous notions about how long *B. bassiana* takes to penetrate the host cuticle. This experiment could help to confirm the results observed for the *psh* line.

Moving forward we are interested in testing more Toll pathway mutants. Since our working hypothesis for this experiment was that the Toll pathway was responsible for sexual dimorphism in fungal infection survival, testing the different genes in this pathway would help to further support our hypothesis if results are replicable amongst the different knockouts. Currently we are in the process of data analysis for a spaezle (*spz*) knockout line.

In this experiment, one of the IMD pathway mutant lines (*rel*) showed no significant difference in male and female survival over 10 days after *B. bassiana* inoculation. This result suggest that *rel* plays an important role in fighting off *B. bassiana* infection in *Drosophila*. This result came as a surprise since it is well documented in the literature that the activation of the IMD signaling pathway is mediated mainly through Gram (-) bacteria and some Gram (+) bacilli

(Nation, 2008, Chapter 15), but there is little evidence to suggest the IMD pathway's involvement in the innate immune response to fungal infection. The purpose of the RNAi crosses was to confirm that the sexual dimorphism observed was due to the deficiency of the gene in this immune response pathway as these were inducible knockout and there would have been no developmental consequences. The results obtained from the induced *rel* knockout demonstrated that sexual dimorphism disappeared in this line, and not in the control line confirming the mutant results. A previous study by Hedengren et al. 1999 demonstrated that Relish is necessary for the induction of the humoral immune response, including antibacterial and antifungal peptides. My results suggest that some genes in the IMD pathway do play a role in the innate immune response to fungal infection in *Drosophila*. Currently we are collecting data for another IMD pathway mutant line (PGRP-LE) in order to further test the IMD pathway as being involved in sexual dimorphism in response to fungal infection with *B. bassiana*. The *imd* mutant line also showed no significant difference in male female survival post inoculation and we are currently collecting data from imd RNAi crosses in order to confirm this result. Similar to our plans for the Toll mutant lines we will also be repeating the experiment with microinjections to see if bypassing the cuticle has an effect on male and female survival to *B. bassiana* infection. If the results are replicated it would provide further evidence to support the novel results observed in this experiment.

The experiments described here originated from a much broader scientific question: what is the genomic basis for evolved resistance to *B. bassiana* in the genetic model *D. melanogaster*? In order to answer this question we must also be able to answer the questions of "How does fungal resistance evolve?" as well as "What genes are involved in fungal immunity?" The first question can be addressed with an artificial selection experiment. One of my colleagues (Yonathan Estrella, Cornell University) and I are working on answering this question using an evolve and resequence

approach. This selection experiment began with four fly populations and their paired controls and utilized *B. bassiana* to create a strong selective pressure. In doing so, we were able to select for high resistance individuals generation after generation and then resequence the genomes of flies from the populations that evolved increased resistance and their control populations in order to observe what mechanisms are at play to enhance *Drosophila's* ability to fight off fungal infection in response to a constant selective pressure. An understanding of sexual dimorphism in response to *B. bassiana* would help to further understand the evolution of fungal resistance in *Drosophila.* In order to answer the question of "What genes are involved in fungal immunity" another colleague of mine is using fully sequenced recombinant inbred lines in order to assay for resistance to *B. bassiana* and using Quantitative Trait Loci analysis to identify potential key genes that underlie immune resistance in *Drosophila* (Kelly Andrés García, Honors Thesis 2015). The experimental design described in my project would then be used to test these new candidate genes and observe their impact on fungal immunity in *D. melanogaster*.

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